Separation of Cholesterol, and Fatty Acylglycerols, Acids and Amides by Thin-Layer Chromatography[1]

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A rapid unidimensional thin-layer chromatographic (TLC) method for the separation of neutral lipids is described, using two sequential solvent systems of different polarity. Excellent separations of mono-, di- and triglycerides, fatty acids, fatty amides, and cholesterol are thereby achieved. Separation is accomplished at room temperature and requires 25 min.
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In the course of our investigations on the modification and hydrolysis of natural lipid mixtures, we found it necessary to rapidly separate and detect fatty acids, fatty amides, mono-, di, and triacylglycerols, as well as cholesterol. Numerous reports (1–9) describe the separation of several of these compounds from each other using thin-layer chromatography (TLC). However, none of the methods was capable of separating all of these compounds from one another. We describe here a TLC method that can achieve this goal. The procedure is efficient, rapid, and reproducible and achieves excellent resolution for both analytical and preparative applications.

MATERIALS AND METHODS

Solvents. Toluene was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Methanol and n-hexane were from Burdick and Jackson (Muskegon, MI). Ethyl acetate and formic acid were from Eastman Kodak Co. (Rochester, NY). Acetone, diethyl ether, chloroform and sulfuric acid were from Mallinckrodt (Paris, KY). Acetic acid was from Aldrich Chemical Co. (Milwaukee, WI). Lipid standards were from Sigma Chemical Co. (St. Louis, MO). *N*-Butylpalmitamide was prepared according to the Krafft-Stauffer procedure (10). Silica Gel G TLC plates (20 × 20 cm, 250 μm) were obtained from Analtech (Newark, DE).

Thin-layer chromatography. Stock solutions were prepared by dissolving 25 mg of each of the reference compounds in 2.5 mL of chloroform. Part of the stock solutions were combined to prepare a standard mixture containing triolein, 1,3-diolein, 1,2-diolein, 1-monoolein, oleic acid, *N*-butylpalmitamide and, as required, cholesterol, each at a concentration of 1.67 mg/mL. TLC plates were washed in a tank of methanol for 5 min. After removal, plates were air-dried for at least 30 min. Samples were applied with a Hamilton syringe 2 cm from the lower edge of the plates. Plates were developed sequentially with solvent A (toluene:diethyl ether:ethyl acetate:acetic acid, 75:10:13:1.2, by vol) to 8 cm above the level of application and dried under nitrogen. The TLC plates were then developed with solvent B (hexane:diethyl ether:formic acid, 80:20:2, v/v/v) to 14 cm above the level of application. After air-drying of the plates, the samples were made visible by spraying the TLC plates with 60% aqueous sulfuric acid and charring on a hot plate.

RESULTS AND DISCUSSION

With the dual development system described in Materials and Methods, the compounds under examination are separated on the basis of polarity, with triacylglycerols exhibiting the greatest mobility, followed by fatty acids (which are protonated in the acidic solvent systems), diacylglycerols, fatty amides, and, finally, monoacylglycerols. Figure 1 illustrates the need for dual solvent development to separate these components. As shown in lane 1 of Figure 1, the attempted separation of individual components with solvent system B alone produced very poor results. Two of the components remained unresolved and another (1-monoacylglycerol) remained at the origin. With solvent system A (Fig. 1, lane 2), all acylglycerols had greater mobility. However, oleic acid and 1,3-diolein

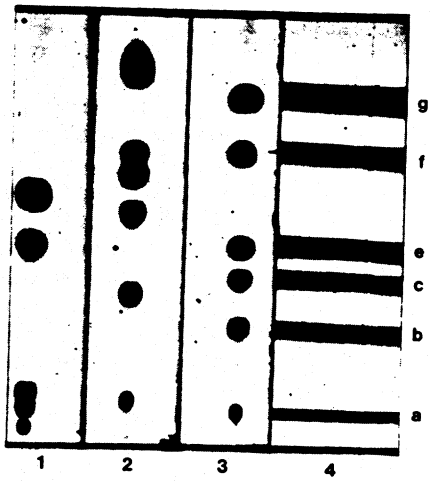

FIG. 1. Thin-layer chromatography of a standard mixture. Lane 1—standard mixture, 25 μg of each component, developed in solvent system B; lane 2—standard mixture, 25 μg of each component, developed in solvent system A; lane 3—standard mixture, 25 μg of each component, developed sequentially in solvent systems A and B; lane 4—preparative TLC of standard mixture, 1.67 mg of each component per plate, developed sequentially in solvent systems A and B; for lanes 3 and 4: a, 1(3)-monoolein; b, *N*-n-butyl palmitamide; c, 1,2-diolein; e, 1,3-diolein; f, oleic acid; g, triolein.

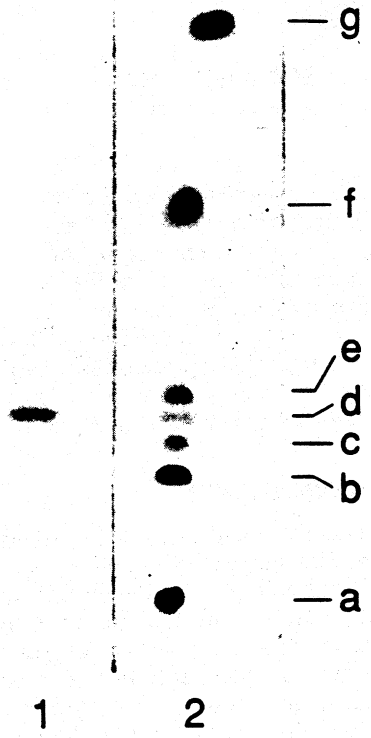

FIG. 2. Separation and detection of cholesterol by sequential development with solvent systems A and B. Lane 1—cholesterol, 25 μg; lane 2—standard mixture, 25 μg each component. a, monoolein; b, *N*-n-butylpalmitamide; c, 1,2-diolein; d, cholesterol; e, 1,3 diolein; f, oleic acid; g, triolein.

were not separated (lane 2). By sequentially using the two solvent mixtures, A and B, excellent separation of the acylglycerols, fatty acid and fatty amide was achieved (Fig. 1, lane 3). Individual components were well resolved from one another and migrated as tight, circular entities. The relative mobilities and the extent of separation of these compounds are unaffected by chain length (at least between C_8 and C_{22}) and degree of unsaturation (up to at least five double bonds; data not shown). However, hydroxylated fatty acids, and acylglycerols containing them, do not exhibit the characteristic mobilities reported here. For example, ricinoleic acid (12-hydroxy-(9Z)-octadecenoic acid) migrates to a level just below that of 1,2-diacylglycerols. Castor oil, which is rich in triacylglycerols containing ricinoleic acid, exhibits two prominent species, one just below ricinoleic acid, and the other just above the 1,2-diacylglycerol fraction. In this system, fatty acid methyl esters and triacylglycerols migrate together.

Successful separations were achieved when the TLC technique described here was used for preparative-scale separations of the components of the standard mixture: lane 4 of Figure 1 illustrates the separation of more than 1.5 mg of each of the components of the standard mixture. We have also found this method useful for the quantitation of individual lipid components, which can be achieved by means of a TLC plate scanner after the plates have been sprayed with sulfuric acid and charred. Alternatively, spraying the preparative plates with an ethanolic solution of 2,7-dichlorofluorescein, visualizing the bands under ultraviolet light, scraping the fractions from the plate and extracting and weighing each fatty component gives quantitative results (data not shown).

The separation of sterols in crude lipid mixtures from the glyceride components is often difficult. Figure 2 illustrates the effectiveness of the method described here in resolving cholesterol from acylglycerols and free fatty acids. As shown in lanes 1 and 2, the mobility of cholesterol is intermediate to that of 1,3- and 1,2-diacylglycerol. The detection and identification of this compound is facilitated by the fact that when the plate is sprayed with aqueous sulfuric acid and then heated for 2–5 min a very bright pink color arises from cholesterol. With further heating, the spot turns black. The separation of cholesterol from diacylglycerols is critically dependent upon the height to which solvent mixture A is allowed to rise on the TLC plate. The optimal development height (usually between 6 and 10 cm) is dependent upon the materials used, and must be determined by trial and error using standard compounds.

The two-solvent, unidimensional thin-layer chromatographic procedure described here is a rapid means for identifying components of fatty mixtures containing mono-, di- and triacylglycerols, fatty acids, fatty amides and cholesterol. The procedure uses inexpensive, readily available solvents and simple laboratory equipment, and results are obtained in a short period of time. We have found this technique to give satisfactory and highly reproducible separations of the components of a wide range of natural lipid mixtures, and to be useful in studies of the hydrolysis and other modifications of these materials.

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